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(54) **VACCINES**

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(58) **Field of Classification Search**
None
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(57) **ABSTRACT**

The invention relates to use of an antigen derived from the circumsporozoite protein (CS) protein of *Plasmodium falciparum* which is expressed at the pre-erythrocytic stage of malarial infection in combination with a pharmaceutically acceptable adjuvant, in the manufacture of a medicament for vaccinating infants against malaria.

15 Claims, 8 Drawing Sheets

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Figure 1. Study design of primary efficacy endpoints

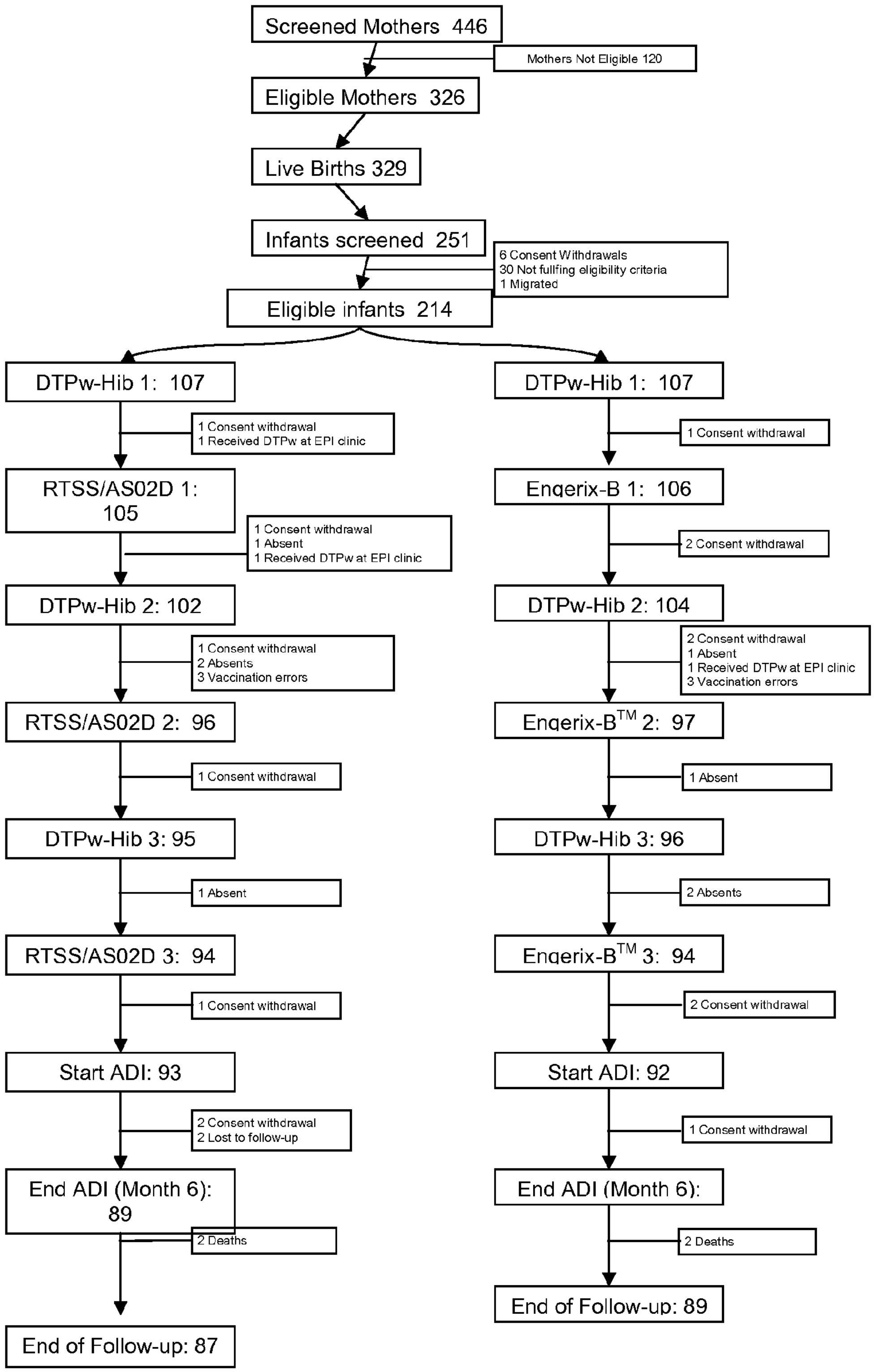


Figure 1a: Trial design

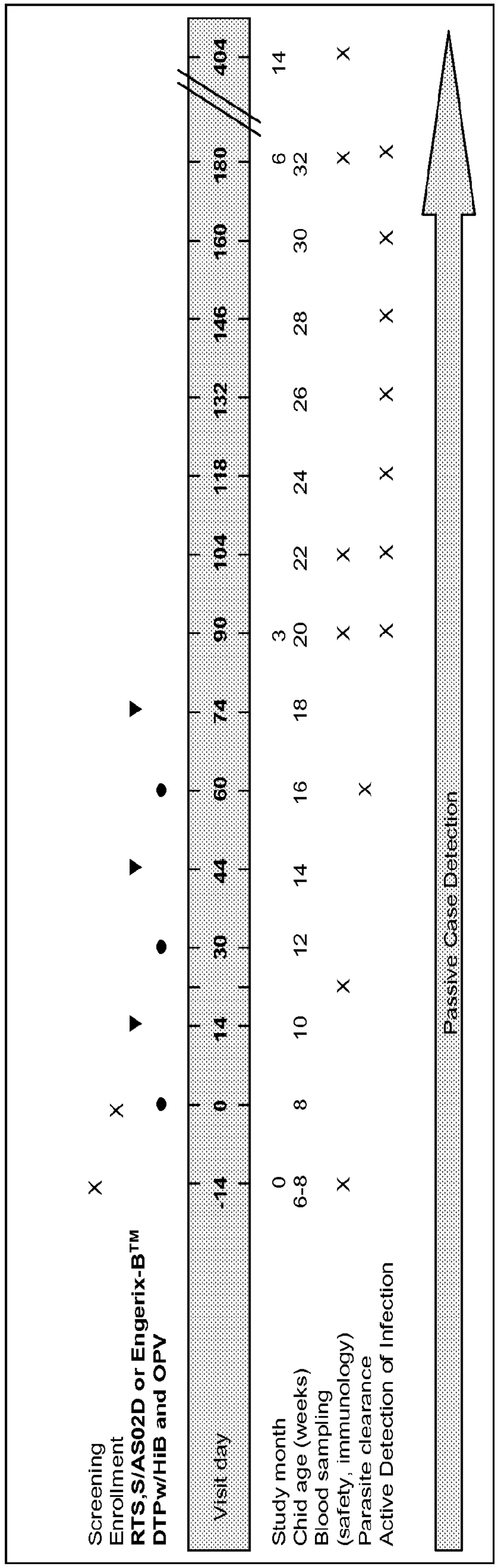


Figure 2a
Reactogenicity data

The graphs below represent solicited systemic and local reactions following vaccination.
R stands for RTS,S
E stands for Engerix-B™.

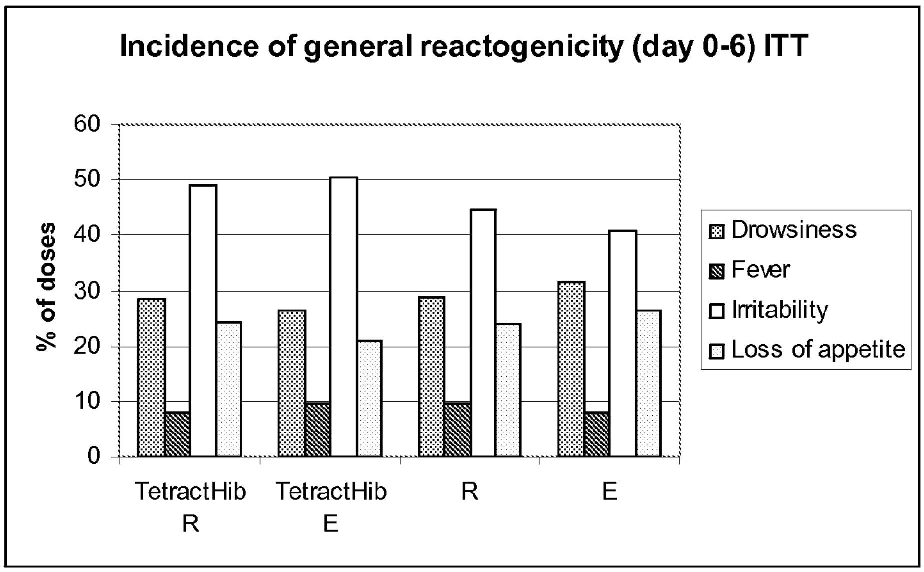


Figure 2b

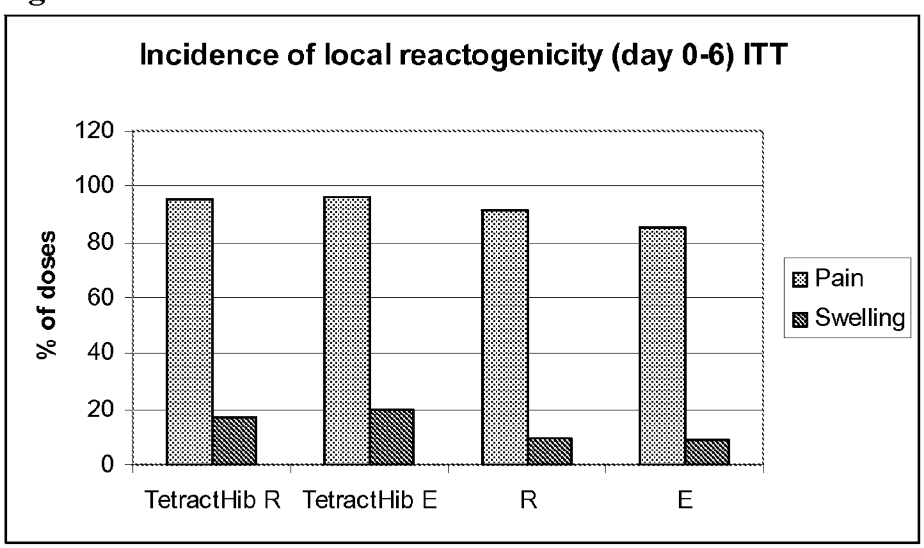


Figure 2c: Proportion of doses with solicited general symptoms reported during the 7 days post vaccination period

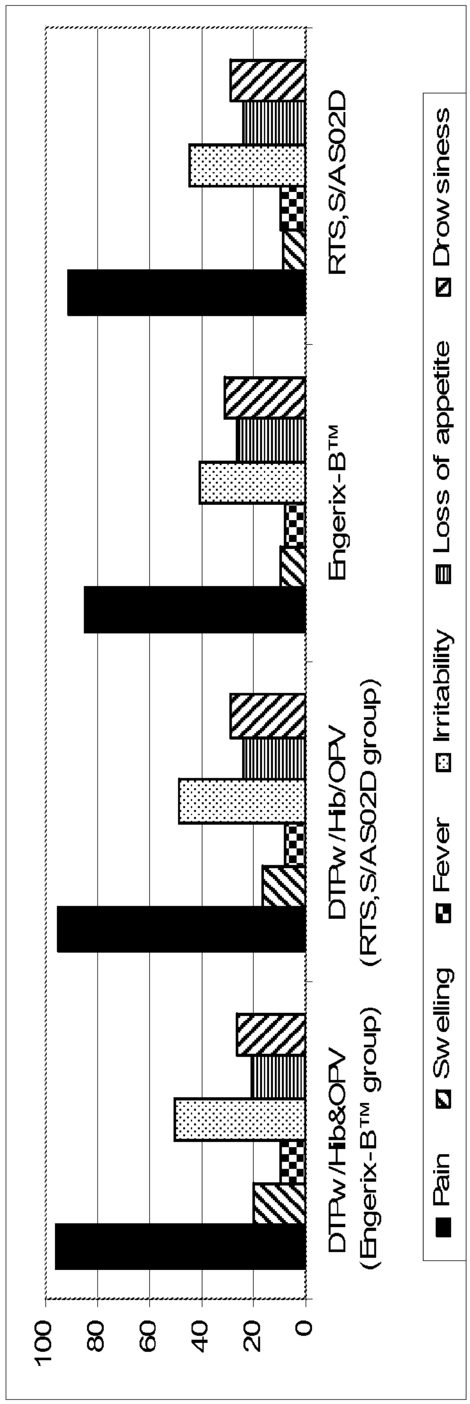


Figure 3

Efficacy

	RTS,S/AS02D				Engerix-B				Point estimate of VE adjusted for covariates				Point estimate of VE unadjusted for covariates			
	Subjects (N)	No. of events	PYAR	Rate	Subjects (N)	No. of events	PYAR	Rate	(%)	95% CI		P value	(%)	95% CI		P value
Infection [a]	93	22	21.82	1.008	92	46	17.22	2.671	65.943	42.600	79.794	<0.001	62.238	37.120	77.322	<0.001
Disease 1 [b]	93	9	22.61	0.398	92	22	19.56	1.125	65.824	25.293	84.366	0.007	64.432	22.646	83.646	0.009
Disease 2 [c]	93	17	22.36	0.760	92	35	18.19	1.924	63.143	33.568	79.552	<0.001	60.976	30.213	78.178	0.002
Disease 3 [d]	107	25	48.51	0.515	107	36	46.02	0.782	38.798	-2.215	63.355	0.061	35.475	-7.535	61.282	0.093

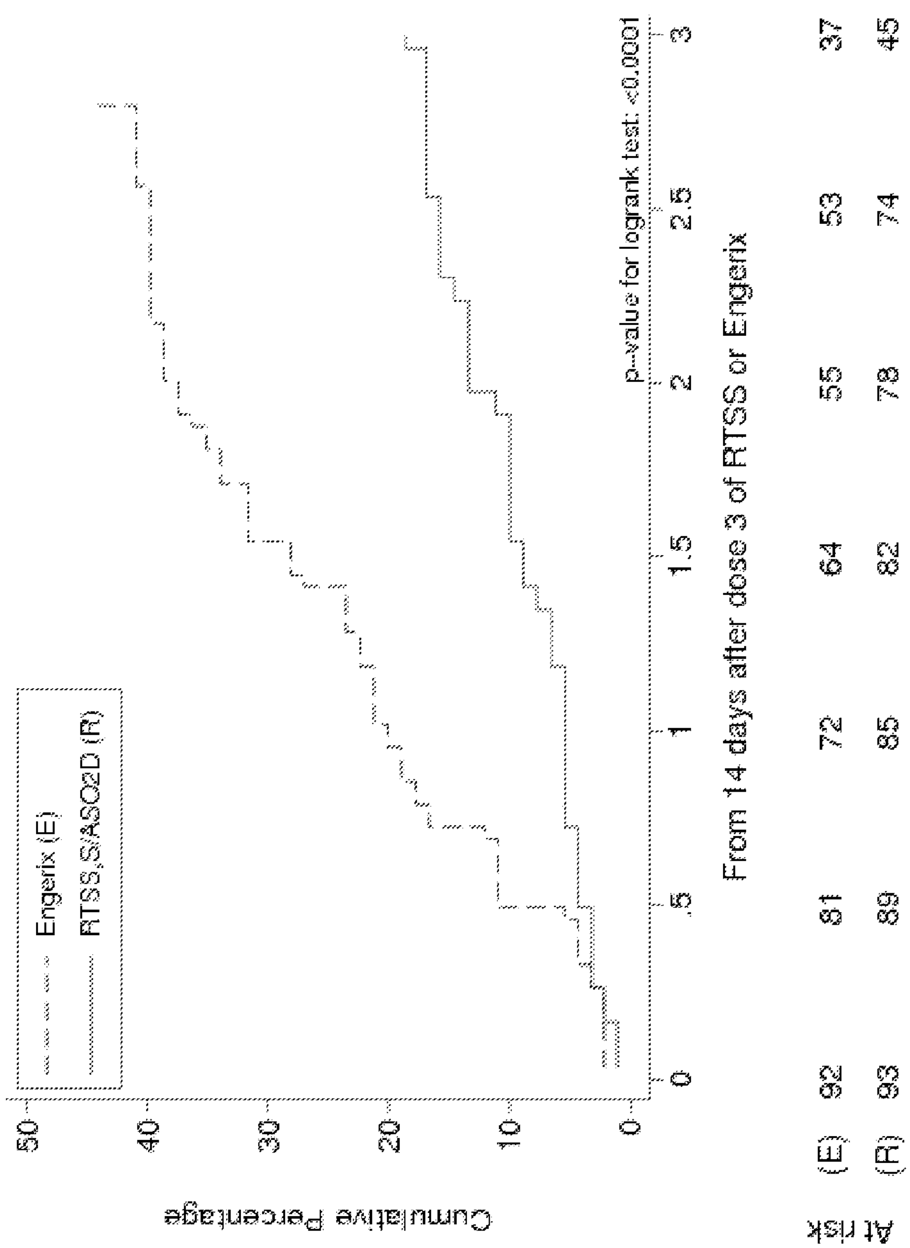


Figure 4: Kaplan-Meier curves for cumulative proportion with at least one episode of malaria infection from 038 trial

Figure 5a. Kaplan-Meier curve for the proportion of children with at least one episode of clinical malaria from the 026 study (children 1-4 in Mozambique)

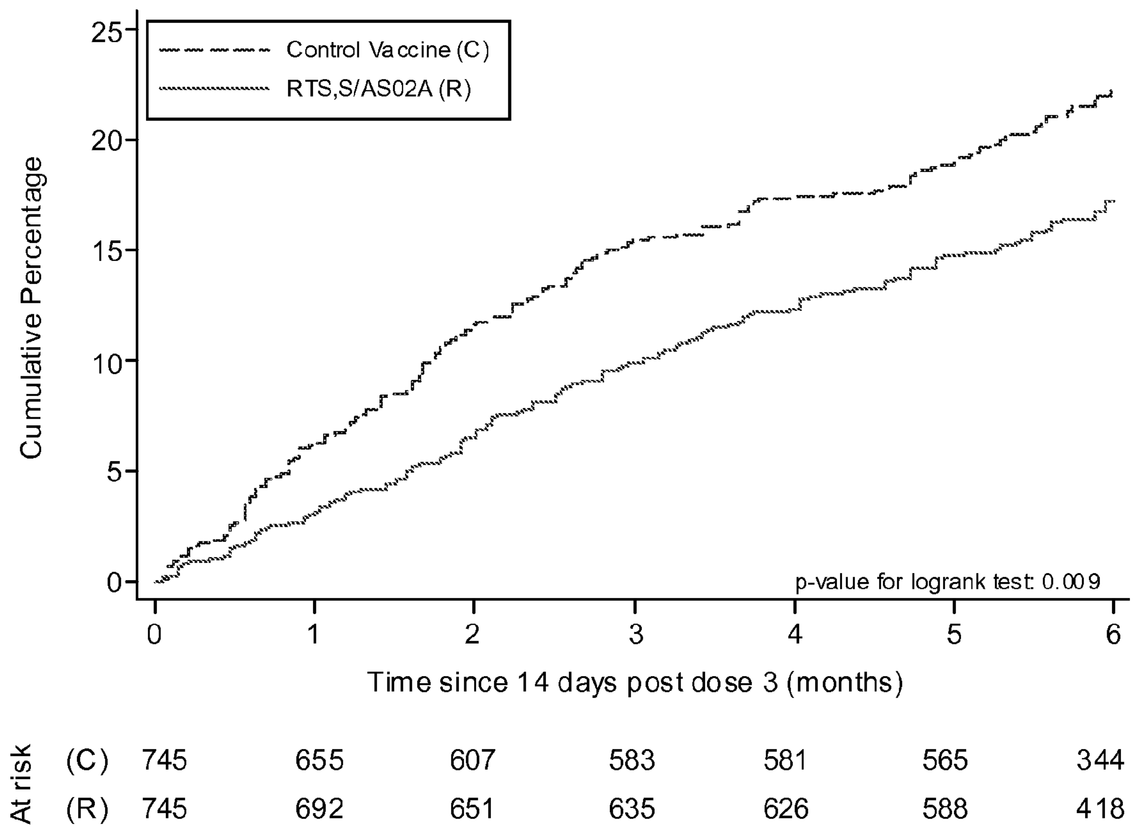
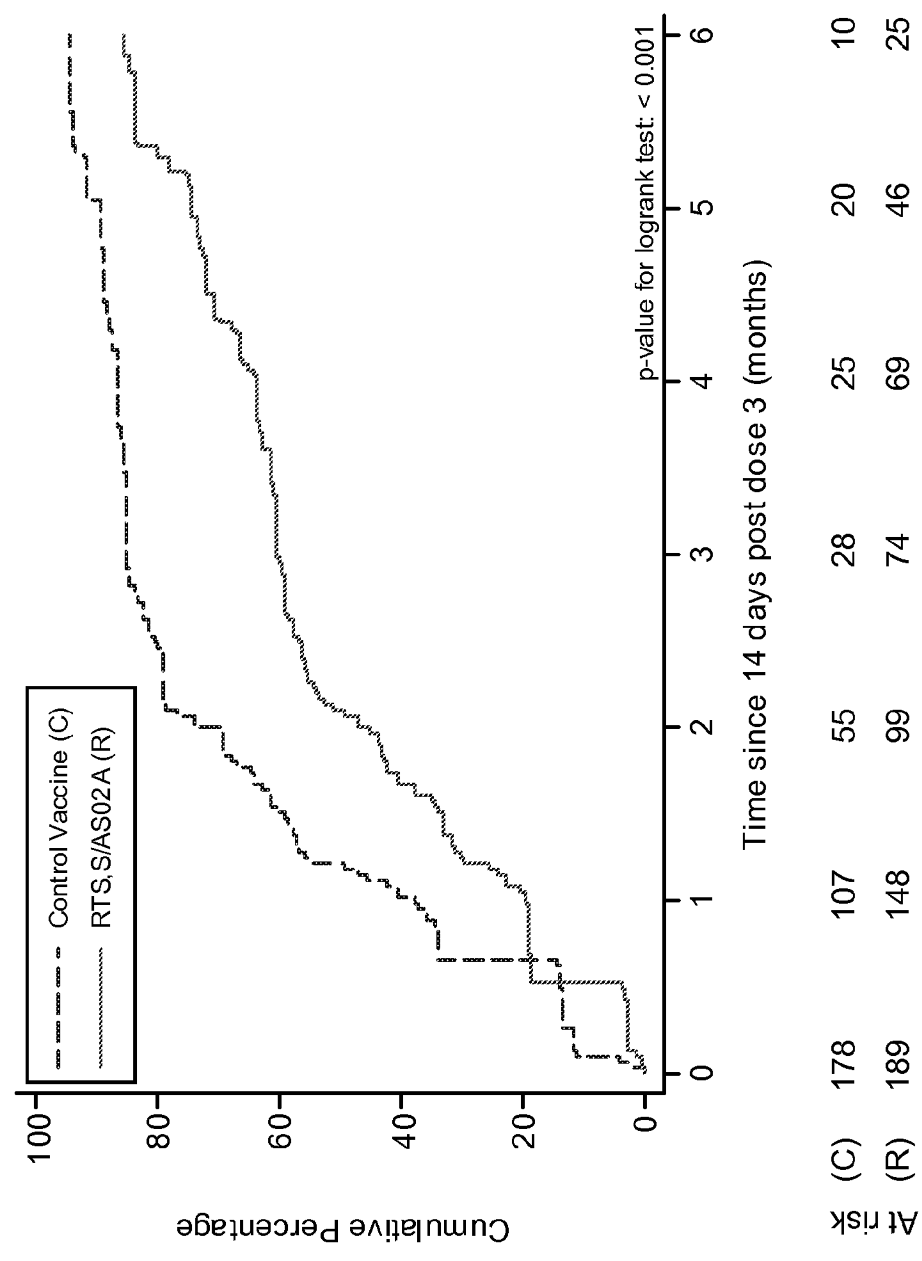


Figure 5b. Kaplan-Meier curve for the proportion of children with at least one episode of malaria infection from the 026 study (children 1-4 in Mozambique)



VACCINES

This application is the US National Stage of International Application No. PCT/EP2008/060505, filed 11 Aug. 2008, which claims benefit of the filing dates of U.S. Provisional Applications No. 60/955,445, filed 13 Aug. 2007, No. 60/957,338, filed 22 Aug. 2007, and No. 60/982,801, filed 26 Oct. 2007, each of which is incorporated herein by reference in its entirety.

The present invention relates to a novel use of a malaria antigen to immunise against malarial disease. The invention relates in particular to the use of circumsporozoite (CS) protein from *P. falciparum* or fragments thereof, to immunise infants against malarial disease.

Malaria is one of the world's major health problems. During the 20th century, economic and social development, together with anti malarial campaigns, have resulted in the eradication of malaria from large areas of the world, reducing the affected area of the world surface from 50% to 27%. Nonetheless, given expected population growth it is projected that by 2010 half of the world's population, nearly 3.5 billion people, will be living in areas where malaria is transmitted[1]. Current estimates suggest that there are well in excess of 1 million deaths due to malaria every year, and the economic costs for Africa alone are thought to be staggering and in the region of at least several billion US dollars annually[2].

These figures highlight the global malaria crisis and the challenges it poses to the international health community. The reasons for this crisis are multiple and range from the emergence of widespread resistance to available, affordable and previously highly effective drugs, to the breakdown and inadequacy of health systems to the lack of resources. Unless ways are found to control this disease, global efforts to improve health and child survival, reduce poverty, increase security and strengthen the most vulnerable societies will fail.

One of the most acute forms of the disease is caused by the protozoan parasite *Plasmodium falciparum* which is responsible for most of the mortality attributable to malaria.

The life cycle of *Plasmodium* is complex, requiring two hosts, man and mosquito for completion. The infection of man is initiated by the introduction of sporozoites from the saliva of a biting and infected mosquito. The sporozoites migrate to the liver and there infect hepatocytes where they differentiate and multiply, via the exoerythrocytic intracellular stage, into the merozoite stage which infects red blood cells (RBC) to initiate cyclical replication in the asexual blood stage. The cycle is completed by the differentiation of a number of merozoites in the RBC into sexual stage gametocytes, which are ingested by the mosquito, where they develop through a series of stages in the midgut to produce sporozoites which migrate to the salivary gland.

The sporozoite stage of *Plasmodium* has been identified as a potential target of a malaria vaccine. Vaccination with inactivated (irradiated) sporozoite has been shown to induced protection against experimental human malaria (Am. J, Trop. Med. Hyg 24: 297-402, 1975). However, it is has not been possible practically and logistically to manufacture a vaccine for malaria for the general population based on this methodology, employing irradiated sporozoites.

The major surface protein of the sporozoite is known as circumsporozoite protein (CS protein). It is thought to be involved in the motility and invasion of the sporozoite during its passage from the initial site of inoculation by the mosquito into the circulation, where it migrates to the liver.

The CS protein of *Plasmodia* species is characterized by a central repetitive domain (repeat region) flanked by non-repetitive amino (N-terminus) and carboxy (C-terminus) fragments. The CS protein in *P. falciparum* has a central repeat region that is highly conserved.

Several groups have proposed subunit vaccines based on various forms or parts of the circumsporozoite protein. Two of these vaccines based exclusively on the central repeat sequence have undergone clinical testing in the early 1980's; one is a synthetic peptide, the other is a recombinant protein (Ballou et al Lancet: Jun. 6 (1987) page 1277 onwards, and Herrington et al Nature 328:257 (1987)). These vaccines were successful in stimulating an anti-sporozoite response. Nonetheless, the magnitude of the response was disappointing, with some vaccinees not making a response at all. Furthermore, the absence of "boosting" of antibody levels after subsequent injections and results of in vitro lymphocyte proliferation assays suggested that T-cells of most of these volunteers did not recognise the immuno-dominant repeat. Furthermore the efficacy of these two vaccines was marginal with only one vaccinated volunteer failing to develop parasitemia. These vaccines therefore were not pursued any further.

WO 93/10152 and WO 98/05355 describe a vaccine derived from the CS protein of *P. falciparum* and it clear that there has been some progress made towards the vaccination against *P. falciparum* using the approach described therein, see also Heppner et al. 2005, Vaccine 23, 2243-50.

The CS protein from *P. falciparum* has been cloned, expressed and sequenced for a variety of strains for example the NF54 strain, clone 3D7 (Caspers et al., Mol. Biochem. Parasitol. 35, 185-190, 1989). The protein from strain 3D7 is characterised by having a central immunodominant repeat region comprising a tetrapeptide Asn-Ala-Asn-Pro repeated 40 times but interspersed with four minor repeats Asn-Val-Asp-Pro. In other strains the number of major and minor repeats varies as well as their relative position. This central portion is flanked by an N and C terminal portion composed of non-repetitive amino acid sequences designated as the repeatless portion of the CS protein.

GlaxoSmithKline Biologicals' RTS,S malaria vaccine based on CS protein has been under development since 1987 and is currently the most advanced malaria vaccine candidate being studied[4]. This vaccine specifically targets the pre-erythrocytic stage of *P. falciparum*, and confers protection against infection by *P. falciparum* sporozoites delivered via laboratory-reared infected mosquitoes in malaria-naïve adult volunteers, and against natural exposure in semi-immune adults[5,6].

RTS,S/AS02A (RTS,S plus adjuvant system) was used in consecutive Phase I studies undertaken in The Gambia involving children aged 6-11 and 1-5 years, which confirmed that the vaccine was safe, well-tolerated and immunogenic[7]. Subsequently a pediatric vaccine dose was selected and studied in a phase I study involving Mozambican children aged 1-4 years where it was found to be safe, well tolerated and immunogenic[8].

WO 2006/029887 describes use of RTS,S to treat severe malaria in children 1 to 4 years of age.

Severe malaria disease is described in the WHO guide to clinical practice (Page: 4 World Health Organization. Management of severe malaria, a practical handbook. Second edition, 2000. http://mosquito.who.int/docs/hbsm.pdf). Classification of children according to the WHO-based definition for severe malaria identifies children who are very sick and at high risk of dying. High risk may be taken to mean about a 30% or greater risk dying.

In the 026 clinical study children aged 1-4 in Mozambique the overall efficacy of the vaccine against infection was calculated to be in the range 45% as measured as a delay in the time to on set of detection of parasites in the blood of a relevant child. The percentage protection against clinical disease was in the range of 35% and the percentage protection against severe malaria disease was in the range of 50%. This level of efficacy was found to persist for a period of about 18 months. (Alonso et al Lancet: 204, 364, page 1411-1420 205, and Lancet 366 pages 2012-2018.)

In the 026 trial there was no evidence of an interaction between age and general vaccine efficacy against non-severe clinical malaria disease, suggesting that efficacy did not significantly change with increasing age. However further exploratory subgroup analysis was carried out to estimate vaccine efficacy in the younger age groups that carry the brunt of malaria disease.

Interestingly this sub-analysis seemed to suggest that efficacy against severe malaria may have been higher in younger children in that trial. There was no suggestion from this analysis that the protection against infection or general efficacy i.e. retarding the development of clinical malaria symptom generally was better in the younger age group.

As far as the inventors are aware only one malaria vaccine has been given to infants under the age of 1 for a number of reasons, including potential toxicity concerns of experimental formulations containing novel adjuvants and/or theories that the immune system of infants is immature and thus no effective protection against malarial infection would be elicited by said vaccination. The strong adjuvants employed in malaria formulations, for example comprising QS21 and/or MPL are not generally used in pediatric vaccines. Instead vaccines for infants generally employ the older aluminium salt adjuvants.

However, a trial in Gambian employing a malaria vaccine SPf66 was performed in infants aged 6 to 11 months (Alonso et al Parasite Immunology, 1997: 19: 579-581). In areas of intense transmission in Tanzania SPf66 had shown 31% protection against first attack of malaria in children aged 1 to 4 years. Nevertheless when tested in infants aged 6 to 11 months the results seemed to indicate that the incidence of clinical malaria in children that had received the SPf66 vaccine were higher than those infants who received the control formulation, which was an inactivated polio vaccine. The effect was most marked in infants who had received a high dose of SPf66.

A recent clinical trial (referred to herein as 038 study) was performed in infants some as young as 10 weeks old at first vaccine administration, with some surprising results (see discussion below).

The present invention provides the use of an antigen derived from the CS protein of *Plasmodium falciparum* in combination with a pharmaceutically acceptable adjuvant, in the manufacture of a medicament for vaccinating infants against malaria.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1** shows an outline of the study design for trial 038

FIG. **1***a* shows an outline of the trial design

FIG. **2***a* shows incidence of general reactogenicity

FIG. **2***b* shows incidence of local reactogenicity

FIG. **2***c* shows proportion of doses with solicited general symptoms reported during the 7 days post vaccination

FIG. **3** shows vaccine efficacy data.

FIG. **4** shows a Kaplan-Meier curve for 038 trial

FIG. **5***a* shows a Kaplan-Meier curve for 026 trial (comparison data)

FIG. **5***b* shows a Kaplan-Meier curve for 026 trial (comparison data)

In FIG. **3** the table presents estimates of VE over a follow-up from 14 days post dose 3 of RTSS or Engerix until month 6 (cross-sectional visit) for the ATP cohort. This includes subjects that received at least 3 doses of RTS,S or Engerix-B, clearance drug and have follow-up time in ADI. Both estimates of VE against infection and disease are presented. Protocol Exploratory endpoint evaluating disease over a time-frame from month 0 (day of first vaccination) to month 6 for the ITT cohort is presented Disease 3.

In FIG. **3**:

PYAR: Episodes/Person Years at Risk;

VE: Vaccine Efficacy;

CI: Confidence Interval

[a]time to first episode; the presence of *P. falciparum* asexual parasitemia >0 per μL

[b]time to first episode; the presence of *P. falciparum* asexual parasitemia >500 per μL and the presence of fever ≥37.5° C. in a child who is unwell and brought for treatment

[c]time to first episode; any level of *P. falciparum* asexual parasitemia and the presence of fever ≥37.5° C. or a history of fever within 24 hours in a child who is unwell and brought for treatment

[d]time to first episode; the presence of *P. falciparum* asexual parasitemia >500 per μL and the presence of fever ≥37.5° C. in a child who is unwell and brought for treatment (ITT 0-6)

Adjusted estimates for area and distance from health center

Malaria in the context of this specification is intended to refer to malaria infection (defined below) and/or clinical malaria disease (also defined below).

In one embodiment the vaccination according to the invention provides a reduced risk of malaria infection. In one aspect the calculated reduction in risk of infection after vaccination is at least about 30, 40, 50%, for example 60 such as 65%.

In one embodiment the vaccination according to the invention provides a reduced risk of developing clinical symptoms of malaria. In one aspect the risk of clinical disease after receiving, for example three doses in a 3 month study interval may be reduced by at least about 30, 40, 50%, for example 60 such as approximately 65% following the third dose.

In one embodiment the malaria is non-severe malaria.

In one embodiment the vaccination provides a reduced risk of developing the following clinical disease symptoms: any level of *P. falciparum* asexual parasitemia and the presence of fever ≥37.5° C. or a history of fever within 24 hours.

In one aspect the reduced risk of malaria infection and/or reduced risk of developing clinical symptoms of malaria is assessed over the 3 months after final vaccination.

In the 038 study 3 doses of RTS,S with adjuvant in a pediatric formulation were given to infants at three time points at approximately monthly intervals (or 4 weekly intervals) nominally time points 0, 1 month and then 2 months. The latter regime forms one aspect of the invention.

The vaccine may be administered at appropriate intervals, for example 2, 3, 4 or 5 week intervals such as 4 week intervals as one, two or three doses.

Many of these infants were very likely truly naïve to malarial infection because of virtually no exposure to infected mosquitoes.

No significant safety or toxicity issues were raised and in fact the malarial vaccines in the study showed similar reactogenicity profiles to a commonly used infant vaccine TETRActHib™ from Aventis Pasteur, which contains antigens to at least diphtheria, tetanus, pertusis and also similar reactogenicity to a Hepatitis B vaccine Engerix-B™.

Furthermore and surprisingly the calculated efficacy of the malaria vaccine employed in the 038 study was about 65% (adjusted vaccine efficacy) against clinical malaria (that is general/non-severe) in comparison to the control group (data in Table 3). This efficacy is about 30% higher than the efficacy, observed in older children, against clinical non-severe disease results of the 026 trial, where the efficacy against non-severe malaria was between 30 and 35%. The latter figures reflect efficacy against non-severe clinical malaria symptoms and not efficacy against severe malaria, which was performed as a sub-analysis in the 026 trial. Thus the vaccination of the present invention seems to translate into a reduction of the risk of acquiring non-severe clinical form of malaria in comparison to the control group in this study and furthermore in comparison vaccinated older children in the 026 trial.

Clinical malaria is defined herein as fever greater than or equal to 37.5° C. with an asexual parasitaemia of *P. falciparum* of 500 present per μL of blood or more (for example with a sensitivity and specificity of greater than 90%).

Also surprising was the fact that the percentage protection against infection seen in the 038 trial was about 65% (Table 3), which the inventors believe is unprecedented and unexpected as the comparable result in the 026 trial was about 45%.

The efficacy data in the two trials (038, see Table 3, and 026) was derived from observations periods that differed in length.

Malaria infection in this context is intended to refer to any asexual *Plasmodium falciparum* parasitaemia detected by active detection of infection (ADI) or passive case detection (PCD).

At the three month monitor time point (that is 3 months after last vaccination) less than 20% about 17% of the infants vaccinated with the malaria vaccine in the 038 trial showed any signs of infection in comparison to more than 40% in the group that had received the comparator/control vaccination.

These observations lead the inventors to the conclusion that in fact the optimal age group for vaccination to provide protection against infection with malarial parasites and clinical malaria is infants.

Infant in the context of this specification is under 1 year such as about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50 weeks old. More specifically, about 10, about 14 and/or about 18 weeks old.

In one embodiment a first vaccination is given to an infant at approximately 6 to 10 weeks after birth, such as 10 weeks.

In one embodiment a second vaccination is given to an infant at approximately 10 to 14 weeks after birth, such as 14 weeks.

In one embodiment a third vaccination is given to an infant at approximately 14 to 18 weeks after birth, such as 16-18 weeks.

A boosting vaccination may be provided after the first course vaccination has been administered. This boost may be administered 6 to 24 months after completion of said primary course.

Whilst not wishing to be bound by theory, the evidence available in this 038 trial does not suggest the anti CS titers were significantly different to those observed in the 026 trial, even though a trend toward higher GMT is seen in 038 for data up to 3 months post dose 3.

Thus it may be advantageous to provide the malaria vaccine to naïve infants before significant natural exposure to malarial infection.

Naïve in the context of this specification is intended to mean that the infant has no or very low detectable antibodies to the CS protein, for example as determined by ELISA or that the infant is so young that it is reasonable to believe that it has not experienced infection by *P. falciparum* parasites post natal.

A low level is below the cut off level defined for a relevant anti CS ELISA. See for example Gorden et al J. Infectious Disease 1995; 171:1576-1585 or Stout el al J. Infectious Diseases 1998; 178: 1139-1144 for further information in relation to suitable assays.

In one aspect of the invention at least 65% of the infants treated with the vaccine are naïve, such as 75, 85, 90, 95 or 99%.

After birth it may be advantageous to protect an infant from exposure to malarial infection by spraying homes and living quarters/bedrooms and the like with insecticide such as DDT. Alternative or additional protection can also be provided by bed nets, which may also be treated with suitable insecticide. These steps may assist in keeping the infant naïve until the malaria vaccine can be administered.

Interestingly in the 038 trial there seemed to be a suggestion that there was a correlation between the level of antibodies generated and protection provided by said vaccination. Infants with CS antibody levels in the highest tertile had 71% lower hazard risk of infection than those in the lower tertile.

In one aspect the invention provides a use of the vaccination for generating an appropriate antibody response to a CS protein, for example wherein 80, 85, 90, 95, 96, 97 or 98% of infants vaccinated according to the invention have anti-CS antibodies above the defined cut off limit for relevant ELISA (such as that used by GSK in clinical trials), about one month to about 4 months after receiving the last vaccination, such as one month and 4 months thereafter.

In one embodiment the antibody response is sufficient to provide a reduced risk of malarial infection and/or a reduced risk of clinical malaria in the vaccinated infant.

In an aspect the invention provides use of a vaccine for delaying infections of an infant with malaria parasites and/or delaying the development of clinical symptoms of malaria. The time to the first malaria infection episode or clinical malaria can, for example be measured using a Cox regression model.

The invention is particularly concerned with reducing the incidence of clinical malaria from *P. falciparum*, such as severe and/or mild forms thereof. Nevertheless in one aspect the invention provided protection against clinical symptoms of non-severe malaria.

Non-severe malaria is herein defined as all clinical cases of malaria that are not severe.

The CS antigens according to the invention may be used in conjunction with another antigen selected from any antigen which is expressed on the sporozoite or the pre-erythrocytic stage of the parasite life cycle such as the liver stage, for example liver stage antigen-1 (LSA-1), liver stage antigen-3 (LSA-3), thrombospondin related anonymous protein (TRAP), merozoite surface protein-1 (MSP1) the major merozoite surface protein, and apical merozoite antigen-1 (AMA-1) which has recently been show to be present at the liver stage (in addition to the erythrocytic stage). All of these antigens are well known in the field. The antigen may be the entire protein or an immunogenic fragment thereof. Immunogenic fragments of malaria antigens are well know, for example the ectodomain from AMA-1.

In one embodiment the *P. falciparum* antigen is fused to the surface antigen from hepatitis B (HBsAg). Thus a circumsporozoite (CS) protein antigen suitable for use in the present invention is in the form of a fusion protein with HBsAg. The antigen may be the entire CS protein from *P. falciparum* or part thereof, including a fragment or fragments of the CS protein, which may be fused together.

In one embodiment the CS protein based antigen is in the form of a hybrid protein comprising substantially all the C-terminal portion of the CS protein of *P. falciparum*, four or more tandem repeats of the CS protein immunodominant region, and the surface antigen from hepatitis B (HBsAg). In one aspect the fusion protein comprises a sequence which contains at least 160 amino acids which is substantially homologous to the C-terminal portion of the CS protein.

In particular "substantially all" the C terminal portion of the CS protein includes the C terminus devoid of the hydrophobic anchor sequence. The CS protein may be devoid of the last 12 to 14 (such as 12) amino-acids from the C terminal.

In one embodiment the fusion protein for use in the invention is a protein which comprises a portion of the CS protein of *P. falciparum* substantially as corresponding to amino acids 207-395 of *P. falciparum* 3D7 clone, derived from the strain NF54 (Caspers et al, supra) fused in frame via a linear linker to the N-terminal of HBsAg. The linker may comprise part or all of the preS2 region from HBsAg.

A suitable CS constructs for use in the present invention are as outlined in WO 93/10152. Particularly suitable is the hybrid protein known as RTS as described in WO 93/10152 (wherein it is denoted RTS*) and WO 98/05355, the whole contents of both of which are incorporated herein by reference.

A particularly suitable fusion protein is the fusion protein known as RTS which consists of:

- A methionine-residue, encoded by nucleotides 1059 to 1061, derived from the *Sacchromyes cerevisiae* TDH3 gene sequence. (Musti A. m. et al Gene 1983 25 133-143).
- Three amino acids, Met Ala Pro, derived from a nucleotide sequence (1062 to 1070) created by the cloning procedure used to construct the hybrid gene.
- A stretch of 189 amino acids, encoded by nucleotides 1071 to 1637 representing amino acids 207 to 395 of the circumsporozoite protein (CSP) of *Plasmodium falciparum* strain 3D7 (Caspers et al, supra).
- An amino acid (Gly) encoded by nucleotides 1638 to 1640, created by the cloning procedure used to construct the hybrid gene.
- Four amino acids, Pro Val Thr Asn, encoded by nucleotides 1641 to 1652, and representing the four carboxy terminal residues of the hepatitis B virus (adw serotype) preS2 protein (Nature 280: 815-819, 1979).
- A stretch of 226 amino acids, encoded by nucleotides 1653 to 2330, and specifying the S protein of hepatitis B virus (adw serotype).

In one embodiment the RTS is in the form of immunogenic particles of RTS,S.

The RTS,S construct may for example comprises two polypeptides RTS and S that are synthesized simultaneously and spontaneously form composite particulate structures (RTS,S).

The RTS protein is preferably expressed in genetically engineered yeast cells, most preferably *S. cerevisiae* or *Picha pastoris*. In such a host, RTS will be expressed as lipoprotein particle (also referred to herein as immunogenic particle or a virus like particle). The preferred recipient yeast strain preferably already carries in its genome several integrated copies of a hepatitis B S expression cassette. The resulting strain synthesizes therefore two polypeptides, S and RTS, that spontaneously co-assemble into mixed (RTS,S) lipoprotein particles. These particles, advantageously present the CSP sequences of the hybrid at their surface. Advantageously the ratio of RTS:S in these mixed particles is, for example 1:4.

It is believed that the presence of the surface antigen from Hepatitis B and the formation of the RTS,S particles boosts the immunogenicity of the CS protein portion of the hybrid protein, aids stability, and/or assists reproducible manufacturing of the protein.

Alternatively the hybrid protein may contain an N-terminal fragment from the CS protein of *P. falciparum.*

In one aspect the hybrid protein may comprise one or more repeat units from the central region of *P. falciparum*. For example 1, 2, 3, 4, 5, 6, 7, 8, 9 or more repeat units.

Alternatively the hybrid protein may contain a C-terminal fragment from the CS protein of *P. falciparum.*

Whilst not wishing to be bound by theory it is thought that the N and C terminus and the central repeat fragments may include several T and B cell epitopes.

In recombinant proteins often unnatural amino acids are introduced in the cloning process and are observed in the final expressed protein. For example several such as 1, 2, 3, 4 or 5 amino acids may be inserted at the beginning (N-terminus) of the protein. If 4 amino acids are inserted at the beginning of the protein they may for example be MMAP. In addition to or alternatively 1, 2, or 3 such as 1 amino acids may be inserted into the body/middle of the protein, provided that the activity and properties, such as immunogenicity, of the protein are not adversely affected.

The use of the malaria vaccine described here may also be in combination with one or more other vaccines commonly administered to infants less than 1 year of age, for example to provide protection against one or more the following: diphtheria, tetanus, pertusis, measles, oral polio and/or Hepatitis B. For example such vaccines may be given at age 1, 2, 3, 4, 5, 6, 7, 8, or 9 months as appropriate such as 6, 10 and 14 weeks. In one aspect said infant vaccine provided is DTPw-Hib optionally with an oral polo vaccine.

The malaria vaccine according to the invention may also be used in combination with a BCG vaccine, for example given 1, 2 or more weeks before the vaccine of the invention.

Vaccines such as oral polio, BCG and/or Hepatitis B vaccine may be given at about 1 week or less post natal.

When the malaria vaccine of the invention is given in conjunction with another one or more infant vaccine then the vaccines may be given simultaneously or about 15, 20, 25 or more days apart. The latter may avoid any adverse interaction of the two or more vaccines.

The invention also relates to a method of treatment comprising administering a therapeutically effective amount of a vaccine as described herein, to infants to provide protection against infection and/or developing clinical malaria.

A suitable vaccination schedule for use in the invention includes the administration of 3 doses of vaccine, at one month intervals. In the alternative embodiments of the invention one, two, three or more doses are employed, given 1, 2, 3, 4, 5, 6, 7, 8 or more weeks apart. Boosting of the same may be employed to complement the above primary course of vaccination.

It may also be appropriate to use general good practice for the prevention of malaria in conjunction with the malaria vaccine described herein. Best practice would include practices such spraying bedrooms etc with insecticides and/or employing a bed net to reduce the exposure of the individual to the mosquitoes. In the 038 trial such practices were used in conjunction with the RTS,S based malaria vaccine.

As the primary endpoint in the 038 trial, clinical episodes of malaria may for example be required to have the presence of *P. falciparum* asexual parasitemia above 500 or above 0 per μL on Giemsa stained thick blood films and the presence of fever (temperature ≥37.5° C.).

The definition for severe malaria may, for example include the presence of one or more of the following: severe malaria anaemia (PCV <15%), cerebral malaria (Blantyre coma score <2) or severe disease of other body systems which could include multiple seizures (two or more generalized convulsions in the previous 24 hours), prostration (defined as inability to sit unaided), hypoglycaemia <2.2 mmol/dL or <40 mg/dL), clinically suspected acidosis or circulatory collapse. These are given in Table 1 below.

Severe malaria anemia	Asexual parasitemia definitive reading Hematocrit <15% No other more probable cause of illness	
Cerebral malaria	Asexual parasitemia definitive reading Coma score ≤2 No other identifiable cause of loss of consciousness	Assess coma score after correction of hypoglycemia and 60 minutes after control of fits. If fitting cannot be controlled within 30 minutes child is included
Severe malaria (other)	Asexual parasitemia definitive reading No other more probable cause of illness Does not meet criteria for severe malaria anemia or cerebral malaria One of the following:	
	Multiple seizures	Two or more generalized convulsions within a 24-hour period prior to admission
	Prostration	Inability to sit unaided
	Hypoglycemia	<2.2 mmol/dL or <40 mg/dL
	Acidosis	Document supportive signs and/or laboratory readouts
	Circulatory collapse	Document supportive signs and/or laboratory readouts

In accordance with the invention, an aqueous solution of the purified hybrid protein may be used directly and combined with a suitable adjuvant or carrier. Alternatively, the protein can be lyophilized prior to mixing with a suitable adjuvant or carrier.

A suitable vaccine dose in accordance with the invention is between 1-100 μg antigen e.g. RTS,S per dose, such as 5 to 75 μg of antigen eg RTS,S, particularly a dose of 25 μg of antigen eg RTS,S protein, for example in 250 to 500 μl (final liquid formulation).

A particularly suitable dose of antigen for pediatric formulations according to the invention is 25 μg, for example in a final volume of 0.5 ml.

In one aspect in accordance with the invention the antigen is combined with an adjuvant or carrier.

Particularly an adjuvant is present, such as an adjuvant which is a preferential stimulator of a Th1 type response.

Suitable adjuvants include but not limited to, detoxified lipid A from any source and non-toxic derivatives of lipid A, saponins and other immunostimulants which are preferential stimulators of a Th1 cell response (also herein called a Th1 type response).

An immune response may be broadly divided into two extreme categories, being a humoral or cell mediated immune response (traditionally characterised by B lymphocytes producing antigen specific antibodies and T lymphocytes acting as antigen specific cellular effectors of protection. TH1 type responses (favouring the cell mediated effector immune responses) and TH2 type response (favouring the induction of antibody response).

Extreme TH1-type immune responses may be characterised by the generation of antigen specific, haplotype restricted cytotoxic T lymphocytes, and natural killer cell responses. In mice TH1-type responses are often characterised by the generation of antibodies of the IgG2a subtype, whilst in the human these correspond to IgG1 type antibodies. TH2-type immune responses are characterised by the generation of a range of immunoglobulin isotypes including in mice IgG1.

It can be considered that the driving force behind the development of these two types of immune responses are cytokines. High levels of TH1-type cytokines tend to favour the induction of cell mediated immune responses to the given antigen, whilst high levels of TH2-type cytokines tend to favour the induction of humoral immune responses to the antigen.

The distinction of TH1 and TH2-type immune responses is not absolute, and can take the form of a continuum between these two extremes. In reality an individual will support an immune response which is described as being predominantly TH1 or predominantly TH2. However, it is often convenient to consider the families of cytokines in terms of that described in murine CD4 positive T cell clones by Mosmann and Coffman (Mosmann, T. R. and Coffman, R. L. (1989) *TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual Review of Immunology,* 7, p 145-173). Traditionally, TH1-type responses are associated with the production of the INF-γ cytokines by T-lymphocytes. Other cytokines often directly associated with the induction of TH1-type immune responses are not produced by T-cells, such as IL-12. In contrast, TH2-type responses are associated with the secretion of IL-4, IL-5, IL-6, IL-10 and tumour necrosis factor-β (TNF-β).

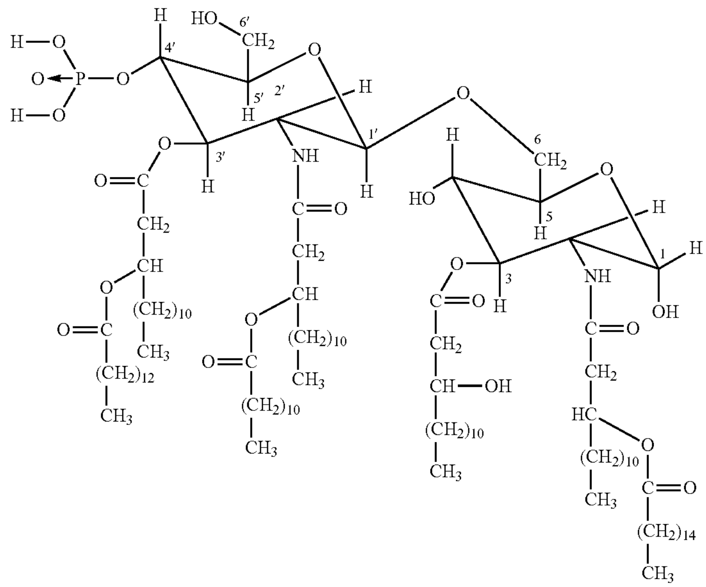
It is known that certain vaccine adjuvants are particularly suited to the stimulation of either TH1 or TH2-type cytokine responses. Traditionally indicators of the TH1:TH2 balance of the immune response after a vaccination or infection includes direct measurement of the production of TH1 or TH2 cytokines by T lymphocytes in vitro after restimulation with antigen, and/or the measurement (at least in mice) of the IgG1:IgG2a ratio of antigen specific antibody responses.

Thus, a TH1-type adjuvant is one which stimulates isolated T-cell populations to produce high levels of TH1-type cytokines when re-stimulated with antigen in vitro, and induces antigen specific immunoglobulin responses associated with TH1-type isotype.

Adjuvants which are capable of preferential stimulation of the TH1 cell response are described in WO 94/00153 and WO 95/17209.

Preferred Th1-type immunostimulants which may be formulated to produce adjuvants suitable for use in the present invention include and are not restricted to the following.

It has long been known that enterobacterial lipopolysaccharide (LPS) is a potent stimulator of the immune system, although its use in adjuvants has been curtailed by its toxic effects. A non-toxic derivative of LPS, monophosphoryl lipid A (MPL), produced by removal of the core carbohydrate group and the phosphate from the reducing-end glucosamine, has been described by Ribi et al (1986, Immunology and Immunopharmacology of bacterial endotoxins, Plenum Publ. Corp., NY, p 407-419) and has the following structure:



A further detoxified version of MPL results from the removal of the acyl chain from the 3-position of the disaccharide backbone, and is called 3-O-Deacylated monophosphoryl lipid A (3D-MPL). It can be purified and prepared by the methods taught in GB 2122204B, which reference also discloses the preparation of diphosphoryl lipid A, and 3-O-deacylated variants thereof.

A preferred form of 3D-MPL is in the form of an emulsion having a small particle size less than 0.2 μm in diameter, and its method of manufacture is disclosed in WO 94/21292. Aqueous formulations comprising monophosphoryl lipid A and a surfactant have been described in WO 98/43670.

The bacterial lipopolysaccharide derived adjuvants to be used in the present invention may be purified and processed from bacterial sources, or alternatively they may be synthetic. For example, purified monophosphoryl lipid A is described in Ribi et al 1986 (supra), and 3-O-Deacylated monophosphoryl or diphosphoryl lipid A derived from *Salmonella* sp. is described in GB 2220211 and U.S. Pat. No. 4,912,094. Other purified and synthetic lipopolysaccharides have been described (Hilgers et al., 1986, *Int. Arch. Allergy. Immunol.,* 79(4):392-6; Hilgers et al., 1987, Immunology, 60(1):141-6; and EP 0 549 074 B1). A particularly preferred bacterial lipopolysaccharide adjuvant is 3D-MPL.

Accordingly, the LPS derivatives that may be used in the present invention are those immunostimulants that are similar in structure to that of LPS or MPL or 3D-MPL. In another alternative the LPS derivatives may be an acylated monosaccharide, which is a sub-portion to the above structure of MPL.

Saponins are also suitable Th1 immunostimulants in accordance with the invention. Saponins are well known adjuvants and are taught in: Lacaille-Dubois, M and Wagner H. (1996. A review of the biological and pharmacological activities of saponins. Phytomedicine vol 2 pp 363-386). For example, Quil A (derived from the bark of the South American tree Quillaja Saponaria Molina), and fractions thereof, are described in U.S. Pat. No. 5,057,540 and "Saponins as vaccine adjuvants", Kensil, C. R., *Crit Rev Ther Drug Carrier Syst,* 1996, 12 (1-2):1-55; and EP 0 362 279 B1. The haemolytic saponins QS21 and QS17 (HPLC purified fractions of Quil A) have been described as potent systemic adjuvants, and the method of their production is disclosed in U.S. Pat. No. 5,057,540 and EP 0 362 279 B1. Also described in these references is the use of QS7 (a non-haemolytic fraction of Quil-A) which acts as a potent adjuvant for systemic vaccines. Use of QS21 is further described in Kensil et al. (1991. J. Immunology vol 146, 431-437). Combinations of QS21 and polysorbate or cyclodextrin are also known (WO 99/10008). Particulate adjuvant systems comprising fractions of QuilA, such as QS21 and QS7 are described in WO 96/33739 and WO 96/11711.

Another immunostimulant is an immunostimulatory oligonucleotide containing unmethylated CpG dinucleotides ("CpG"). CpG is an abbreviation for cytosine-guanosine dinucleotide motifs present in DNA. CpG is known in the art as being an adjuvant when administered by both systemic and mucosal routes (WO 96/02555, EP 468520, Davis et al., *J. Immunol,* 1998, 160(2):870-876; McCluskie and Davis, *J. Immunol.,* 1998, 161(9):4463-6). Historically, it was observed that the DNA fraction of BCG could exert an anti-tumour effect. In further studies, synthetic oligonucleotides derived from BCG gene sequences were shown to be capable of inducing immunostimulatory effects (both in vitro and in vivo). The authors of these studies concluded that certain palindromic sequences, including a central CG motif, carried this activity. The central role of the CG motif in immunostimulation was later elucidated in a publication by Krieg, Nature 374, p 546 1995. Detailed analysis has shown that the CG motif has to be in a certain sequence context, and that such sequences are common in bacterial DNA but are rare in vertebrate DNA. The immunostimulatory sequence is often: Purine, Purine, C, G, pyrimidine, pyrimidine; wherein the CG motif is not methylated, but other unmethylated CpG sequences are known to be immunostimulatory and may be used in the present invention.

In certain combinations of the six nucleotides a palindromic sequence is present. Several of these motifs, either as repeats of one motif or a combination of different motifs, can be present in the same oligonucleotide. The presence of one or more of these immunostimulatory sequences containing oligonucleotides can activate various immune subsets, including natural killer cells (which produce interferon γ and have cytolytic activity) and macrophages (Wooldrige et al Vol 89 (no. 8), 1977). Other unmethylated CpG containing sequences not having this consensus sequence have also now been shown to be immunomodulatory.

CpG when formulated into vaccines, is generally administered in free solution together with free antigen (WO 96/02555; McCluskie and Davis, supra) or covalently conjugated to an antigen (WO 98/16247), or formulated with a carrier such as aluminium hydroxide ((Hepatitis surface antigen) Davis et al. supra; Brazolot-Millan et al., *Proc. Natl. Acad. Sci.*, USA, 1998, 95(26), 15553-8).

Such immunostimulants as described above may be formulated together with carriers, such as for example liposomes, oil in water emulsions, and/or metallic salts, including aluminium salts (such as aluminium hydroxide). For example, 3D-MPL may be formulated with aluminium hydroxide (EP 0 689 454) or oil in water emulsions (WO 95/17210); QS21 may be advantageously formulated with cholesterol containing liposomes (WO 96/33739), oil in water emulsions (WO 95/17210) or alum (WO 98/15287); CpG may be formulated with alum (Davis et al. supra; Brazolot-Millan supra) or with other cationic carriers.

Combinations of immunostimulants are also preferred, in particular a combination of a monophosphoryl lipid A and a saponin derivative (WO 94/00153; WO 95/17210; WO 96/33739; WO 98/56414; WO 99/12565; WO 99/11241), more particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153. Alternatively, a combination of CpG plus a saponin such as QS21 also forms a potent adjuvant for use in the present invention.

Thus, suitable adjuvant systems include, for example, a combination of monophosphoryl lipid A, preferably 3D-MPL, together with an aluminium salt.

An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched in cholesterol containing liposomes (DQ) as disclosed in WO 96/33739.

A particularly potent adjuvant formulation involving QS21, 3D-MPL & tocopherol in an oil in water emulsion is described in WO 95/17210 and is another preferred formulation for use in the invention.

Another preferred formulation comprises a CpG oligonucleotide alone or together with QS21, 3D-MPL or together with an aluminium salt.

Accordingly in one embodiment of the present invention there is provided the use of detoxified lipid A or a non-toxic derivative of lipid A, more preferably monophosphoryl lipid A or derivative thereof such as 3D-MPL, in combination with a malaria antigen as described herein, for the manufacture of a vaccine for the prevention of malaria disease and/or infection in infants.

In one embodiment of the invention a saponin, such as QS21, is used in combination with a malaria antigen as described herein, for the manufacture of a vaccine for the prevention of malaria disease and/or infection in infants or a method of treating an infant employing the same.

In one embodiment the invention provides use of detoxified lipid A or a non-toxic derivative of lipid A, more preferably monophosphoryl lipid A or derivative thereof such as 3D-MPL, in combination with a saponin, such as QS21, and a malaria antigen as described herein, for the manufacture of a vaccine for the prevention of malaria disease and/or infection in infants. The invention also extends to an method of treating an infant employing these aspects.

In one embodiment the invention further employs an oil in water emulsion or liposomes. Suitable combinations of adjuvants for use in the present invention are:

1. 3D-MPL, QS21 and an oil in water emulsion.
2. 3D-MPL and QS21 in a liposomal formulation.
3. 3D-MPL, QS21 and CpG in a liposomal formulation.

The amount of 3D-MPL used is generally small, but depending on the vaccine formulation may be in the region of 1-1000 μg per dose, generally 1-500 μg per dose, such as between 1 to 100 μg per dose (10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80 or 90 μg per dose).

The amount of saponin for use in the adjuvants of the present invention may be in the region of 1-1000 μg per dose, generally 1-500 μg per dose, more such as 1-250 μg per dose, and more specifically between 1 to 100 μg per dose (10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80 or 90 μg per dose).

The amount of CpG or immunostimulatory oligonucleotides in the adjuvants or vaccines of the present invention is generally small, but depending on the vaccine formulation may be in the region of 1-1000 μg per dose, generally 1-500 μg per dose, and more such as between 1 to 100 μg per dose (10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80 or 90 μg per dose).

In one aspect of the invention the dose has a final volume of 0.5 ml.

The vaccines of the invention may be provided by any of a variety of routes such as oral, topical, subcutaneous, mucosal), intravenous, intramuscular, intranasal, sublingual and intradermal.

Immunisation can be prophylactic or therapeutic. The invention described herein is primarily but not exclusively concerned with prophylactic vaccination against malaria, more particularly prophylactic vaccination to prevent or to reduce the likelihood of malarial infection and/or malaria disease.

Appropriate pharmaceutically acceptable carriers or excipients for use in the invention are well known in the art and include for example water or buffers. Vaccine preparation is generally described in Pharmaceutical Biotechnology, Vol. 61 Vaccine Design—the subunit and adjuvant approach, edited by Powell and Newman, Plenum Press New York, 1995. New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Md., U.S.A. 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Pat. No. 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S. Pat. No. 4,372,945 and by Armor et al., U.S. Pat. No. 4,474,757.

In the context of this specification comprising is to be interpreted as including.

Aspects of the invention comprising a certain element are also intended to extend to said aspects consisting or consisting essentially of the relevant elements and vice versa, if appropriate

EXAMPLE

Study Design

The study was conducted at the Centro de Investigação em Saude de Manhiça (CISM, Manhiça Health Research Centre) between June 2005 and March 2007, in Ilha Josina and Taninga, two communities about 50 km north of the town of Manhiça, where CISM headquarters are located. Characteristics of the overall study area and of Ilha Josina have been previously described (Alonso P L, Sacarlal J, Aponte J J, Leach A, Macete E, Milman J, et al. Efficacy of the RTS,S/

AS02A vaccine against *Plasmodium falciparum* infection and disease in young African children: randomised controlled trial. Lancet 2004 Oct. 16-22; 364(9443):1411-20). Taninga is a rural community facing Ilha Jossina directly across the flood plain of the Incomati River. The climate is subtropical with two distinct seasons: a warm and rainy season from November to April and a generally cool and drier season during the rest of the year. Malaria transmission is perennial with some seasonality and mostly attributable to *P. falciparum. Anopheles funestus* is the main vector. Combination therapy based on amodiaquine and sulphadoxine-pirimethamine was the first line treatment for uncomplicated malaria until September 2006 when it was changed to artemisinin based combination therapy (ACT) using artesunate and sulphadoxine-pyrimethamine. Since 2005, yearly rounds of indoor residual spraying (IRS) have been carried out as part of the malaria control activities of the Ministry of Health. During the first round in December 2005, IRS was based on Carbamates (ICON®) but this was changed to DDT in December 2006. As part of the study activities and in accordance with national recommendations, an insecticide treated bed net (ITN) was given to pregnant women at screening, along with instructions for its use. Both Ilha Josina and Taninga have primary health posts and maternities that provide basic curative and preventive care. For the study, facilities were upgraded, around the clock care was available, and transport provided for referrals to the Manhica District Hospital, adjacent to CISM.

The study was a phase I/IIb double blind randomized controlled trial to assess the safety, immunogenicity and efficacy test of concept of the RTS,S/AS02D candidate malaria vaccine when administered to infants by immunisations given at 10, 14 and 18 weeks of age. The protocol was approved by the Mozambican National Bioethics Committee, the Hospital Clinic of Barcelona Ethics Review Committee and the PATH Human Subjects Protection Committee. Trial registration number is NCT00197028 and IND number is BB-IND 10514. The trial was undertaken according to the International Conference of Harmonization Good Clinical Practices guidelines and was monitored by GSK Biologicals. A Local Safety Monitor and a Data and Safety Monitoring Board closely reviewed the design conduct and results of the trial.

Participants

CISM operates a demographic surveillance system in about half of the district which includes both study locations. Pregnant women in their third trimester of pregnancy, resident in the study area, who would consider enrolling their infants, were asked to take part in the informed consent process. At first visit, an information sheet was read and explained to groups of pregnant women by specially trained staff. We sought individual consent only after the women passed an individual oral comprehension test designed to check understanding of the information. They were invited to sign (or thumbprint if not literate) the informed consent document. A member of the community, not associated with the research study, acted as an impartial witness and countersigned the consent form. Those that gave informed consent received counselling and were screened for HIV (Determine™ HIV1-2, Abbot Laboratories and UNI-GOLD HIV, Trinity Biotech PLC) and Hepatitis B (Determine™ HBsAg, Abbot Laboratories). Women found to be HIV positive were referred to the government health services at the Manhiça District Hospital for medical evaluation and management following National Guidelines. These included reduction of mother to child transmission as well as the free provision of anti-retro viral therapy to those fulfilling clinical and social criteria. Hepatitis B positive mothers were counseled about the risk of transmission to the offspring and Hepatitis B vaccine at birth was offered to the newborn.

Following another informed consent of the mother, and using similar procedures as those previously described, infants were screened between 6 and 12 weeks of age. This included a brief medical history and physical examination and blood sampling by heel prick or venous puncture for baseline haematology, biochemistry and immunology. Inclusion criteria included, among others, a normal gestational period and absence of obvious medical abnormalities. Because children born to Hepatitis B and HIV-positive mothers are at a high risk of neonatal acquisition of the viruses, they were not included in the trial. Among other criteria, children were excluded from participation if BCG was not given at least one week before starting study vaccination or if any other vaccinations, other than the first dose of OPV given at birth with BCG, had been given prior to enrollment.

An individual photographic identification card was provided soon after recruitment. This included the name of the child and the mother as well as the personal identification number from the census (Alonso P, Saute F, Aponte J J. Manhiça DSS, Mozambique. In: Sankoh O A, Ngom P, Nyarko P, Mwageni E, Kahn K, editors. Population and health in developing countries; vol. 1, population, health and survival at INDEPTH sites. Ottawa: International development research center (IDRC); 2002. p. 189-95) and a unique study number issued at the screening visit. Field activities started on 24 Jun. 2005. The first infant was enrolled on 23 Aug. 2005 and the last one on 12 Sep. 2006. Follow-up activities for the double blind phase were completed on Mar. 6, 2007, when the last recruited child reached their 6 month study visit.

Procedures

EPI in Mozambique includes BCG vaccine and Oral Polio Vaccine (OPV) at birth, three doses of Dipheteria-Tetanus-Pertusis whole cell (DTPw) and Hepatitis B vaccines, co-administered with OPV at 8, 12 and 16 weeks of age and measles vaccine at 9 months of age. For the purpose of the study we introduced two changes to this scheme. Given that the use of Hib vaccine is fast expanding and is becoming part of EPI in a growing number of African countries, we decided to include it combined with DTPw as it would provide additional benefit for children participating in the trial. Hepatitis B vaccine was not given together with DTPw as RTS,S has been shown to induce comparable titers of anti-HBsAg. FIG. **1***a* represents the trial design and follow-up scheme. Eligible children were enrolled the day of the first vaccination with DTPw/Hib [TETRActHib™ Aventis Pasteur]. Children were randomly allocated, at the time of the first vaccination with DTPw/Hib, to receive three doses of either RTS,S/AS02D (GSK Biologicals, Rixensart. Belgium) or Hepatitis B vaccine (Engerix-B™, GSK Biologicals, Rixensart, Belgium) staggered by 2 weeks with DTPw/Hib and OPV vaccines and administered at 10, 14 and 18 weeks of age. Block randomization was done at GSK Biologicals with SAS software version 8 (1:1 ratio, block size of 2). The randomization code was released to the investigators once databases had been monitored, cleaned and locked.

The infants randomised to the paediatric formulation of the malaria vaccine group received 0.5 ml of the RTS,S/AS02D formulation containing 25 μg of RTS,S and the Adjuvant System AS02D. RTS,S is a hybrid recombinant protein consisting of the *P. falciparum* CS protein central tandem repeat and carboxy-terminal regions fused to the amino-terminus of the S antigen of hepatitis B virus (HBsAg). The proteins auto-assemble to form a particle that also includes unfused S antigen. Antigen and GlaxoSmithKline's proprietary AS02D Adjuvant System are described in detail elsewhere (Macete E V, Sacarlal J, Aponte J J, Leach A, Navia M M, Milman J, et al. Evaluation of two formulations of adjuvanted RTS, S malaria vaccine in children aged 3 to 5 years living in a malaria-endemic region of Mozambique: a Phase I/IIb randomized double-blind bridging trial. Trials 2007 Mar. 26; 8:11).

DTPw/Hib was administered by the intramuscular (IM) route in the right antero-lateral thigh while RTS,S/AS02D or Engerix-B™ were administered IM in the left antero-lateral thigh. The RTS,S/AS02D or Engerix-B™ vaccination was delivered in a double blind fashion (observer blinded, participant blinded). The vaccination team prepared the vaccine and masked the contents of the syringe with opaque tape before providing the syringe to the clinical team for immunisation of the infant. Since the two vaccines used in the study were of distinct appearance, the vaccination team was not blinded and was not involved in any other study procedure.

After each vaccination, infants were observed for at least one hour. Trained field workers visited the children at home every day for the following six days to record any adverse event (AE). Solicited local and general adverse events were recorded during this period. Unsolicited adverse events were recorded for 30 days after each dose through the health facility based morbidity surveillance system. Serious adverse events (SAEs) were detected in a similar way and recorded throughout the study. A detailed description of definitions for solicited and unsolicited adverse events as well as SAEs can be found elsewhere[11]. Further safety assessment was performed through haematological and biochemical assessments, including renal and hepatic function measured at several time points as shown in FIG. **1***a*. Anti-HBsAg antibody titres were measured at baseline and one month post dose 3 of RTS,S/AS02D or Engerix-B™. Anti-CS antibody titres were measured at baseline, and one and three and a half months post dose 3 of RTS,S/AS02D or Engerix-B™. Antibodies against diphtheria and tetanus toxins and polyribosyl ribitol phosphate (PRP) for *H. influenzae* type b were measured one month after dose 3 of DTPw/Hib. Antibodies against *B. pertussis*, were measured at baseline and one month after the 3rd dose of DTPw/Hib.

Cases of malaria infection by *P. falciparum* were monitored by active detection of infection (ADI) and by passive case detection (PCD) at health facilities in the study area. ADI consisted of repeated visits to study participants at predefined intervals (FIG. **1***a*). At each visit, a blood slide for parasitaemia determination was collected irrespective of the presence or absence of symptoms, and the axillary temperature was recorded. Children with positive slides were treated with first line treatment regardless of the presence of symptoms and excluded from further assessment of ADI. Before starting the ADI follow-up, asymptomatic parasitaemia was cleared presumptively in all children with a combination of amodiaquine (10 mg/kg/day for 3 days) and a single dose of sulfadoxine-pyrimethamine (sulfadoxine 25 mg/kg and pyrimethamine 1.25 mg/kg) administered two weeks prior to the third and final dose of RTS,S/AS02D or Engerix-B™ Parasitaemia was checked 2 weeks later at the time of the third dose and, if present, treated with second-line treatment based on Coartem®. Only children without parasitemia started the ADI, which commenced 2 weeks after the third dose of RTS, S/AS02D or Engerix-B™ and was performed every-other week for 12 weeks.

The health facility based morbidity surveillance system set up at the Manhiça District Hospital, Ilha Josina and Taninga health posts, enabled passive case detection (PCD) of all attendances to health facilities and ascertainment of episodes of clinical malaria. This surveillance system has been described in detail elsewhere (Alonso P L, Sacarlal J, Aponte J J, Leach A, Macete E, Milman J, et al. Efficacy of the RTS,S/AS02A vaccine against *Plasmodium falciparum* infection and disease in young African children: randomised controlled trial. Lancet 2004 Oct. 16-22; 364(9443):1411-20). In brief, the three facilities have 24 h medical staff trained to identify study participants through the personal identification card and to ensure standardized assessment, management and documentation throughout follow-up. All children with documented fever (37.5° C. or more) or history of fever in the preceding 24 h, or pallor had blood taken for parasite and packed cell volume (PCV) determinations. Children meeting admission criteria were referred to the Manhiça District Hospital for hospitalization. Clinical management was provided according to standard national guidelines. On admission and discharge all relevant information was recorded on standardised forms.

To determine parasite presence and density of *P. falciparum* asexual stages, Giemsa stained blood slides were read following standard quality-controlled procedures described elsewhere (Alonso P L, Sacarlal J, Aponte J J, Leach A, Macete E, Milman J, et al. Efficacy of the RTS,S/AS02A vaccine against *Plasmodium falciparum* infection and disease in young African children: randomised controlled trial. Lancet 2004 Oct. 16-22; 364(9443):1411-20). Biochemical parameters were measured using a dry biochemistry photometer VITROS DT II (Orto Clinical Diagnostics, Johnson & Johnson Company, USA). Haematological tests were performed using a Sysmex KX-21N cell counter (Sysmex Corporation Kobe, Japan). PCV was measured in heparinised microcapillary tubes using a Hawksley haematocrit reader after centrifugation.

Antibodies specific for the CS protein tandem repeat epitope were measured by a standard ELISA using plates absorbed with the recombinant antigen R32LR that contains the sequence [NVDP(NANP)15]2LR. The assay cut-off was set at 0.5 EU/ml. Anti-HBsAg antibody levels were measured using a commercial radioimmunoassay (AUSAB, Abbott) with an assay cut-off set at 10 IU/ml. Anti-PRP antibodies were measured by ELISA with a cut-off set at 0.15 μg/ml. Anti-diphtheria and anti-tetanus antibodies titres were measured by ELISA with an assay cut-off set at 0.10 IU/ml. Anti-whole-cell-B. Pertussis antibody titres were determined by ELISA (Labsystems) with an assay cut-off set at 15 EL.U/ml.

Statistical Analysis

The primary endpoints of the trial were safety and tolerability of the RTS,S/AS02D vaccine candidate. All children who received at least one dose of DTPw/Hib were included in the intention-to-treat (ITT) safety analysis.

Anti-CS and anti-HBsAg antibody data were summarised by Geometric Mean Titres (GMTs) with 95% CI. Anti-CS seropositivity was defined as ≥0.5 EU/ml, while seroprotection from Hepatitis B was defined as ≥10 IU/ml.

The test of concept for vaccine efficacy (VE) was based on a prospectively-defined Report & Analysis Plan (RAP) carried out on an According to Protocol (ATP) cohort. The ATP cohort included subjects that met all eligibility criteria, completed the vaccination course and contributed follow-up time during the ADI period. The analysis included all first or only asexual *P. falciparum* infections detected during the follow-up period starting 14 days after dose 3 of RTS,S/AS02D or Engerix-B™ and ending with the visit at study month 6 (approximately a 3 month follow-up). Malaria infections included in this analysis were detected by ADI or PCD.

Further exploratory analysis considered vaccine efficacy against clinical malaria in an ITT cohort that included first or only episodes from the time of enrollment until the date on which the last enrolled infant completed their ADI follow-up (Mar. 6, 2007). Additionally, exploratory analyses of clinical malaria were performed in the same ATP cohort and follow-up period as in the efficacy assessment of new infections. The primary case definition for clinical malaria was fever (axillary temperature ≥37.5° C.) with an asexual parasitaemia of *P. falciparum* of 500 or more per microliter. This definition has a reported sensitivity and specificity of greater than 90% (Saute F, Aponte J, Almeda J, Ascaso C, Abellana R, Vaz N, et al. Malaria in southern Mozambique: malariometric indicators and malaria case definition in Manhica district. Trans R Soc Trop Med Hyg 2003 November-December; 97(6):661-6). Other exploratory efficacy analyses used secondary case definitions for clinical malaria including fever or history of fever in the previous 24 hours plus any asexual *P. falciparum* parasitaemia. The time at risk, reported as person years at risk (PYAR), was adjusted for absences from the study area and for antimalarial drug usage as previously described (Alonso P L, Sacarlal J, Aponte J J, Leach A, Macete E, Milman J, et al. Efficacy of the RTS,S/AS02A vaccine against *Plasmodium falciparum* infection and disease in young African children: randomised controlled trial. Lancet 2004 Oct. 16-22; 364 (9443):1411-20), as these are periods of time when infants could not be expected to contribute study endpoints.

VE was defined as 1 minus the rate ratio. Vaccine efficacy was adjusted by distance to health facility, calculated according to previously described methods (Alonso P L, Sacarlal J, Aponte J J, Leach A, Macete E, Milman J, et al. Efficacy of the RTS,S/AS02A vaccine against *Plasmodium falciparum* infection and disease in young African children: randomised controlled trial. Lancet 2004 Oct. 16-22; 364(9443):1411-20) and community of residence. The adjusted vaccine efficacy, assessed using Cox regression models, is reported throughout the text unless otherwise stated. The effect of anti-CS antibody titres on the risk of malaria infection was evaluated in the group that received RTS,S/AS02D by comparing the hazard ratio (HR) of infants in the lowest response tertile against those in the highest response tertile, as well as estimating the HR per doubling of anti-CS antibody titre using Cox regression models. Finally, a comparison was made of the geometric mean titre of children who had at least one episode of malaria infection against those without documented malaria infection using a Wilcoxon Rank Sum test.

The sample size was based on the evaluation of vaccine safety. A trial with 100 subjects in each group had 80% power to detect a difference in the proportion of adverse events of 26% or more if the frequency of an event in the Engerix-B™ group was 10% or more. A trial of this size also had 90% power to detect an efficacy against malaria infection of 45% or more assuming an attack rate of 75% or more in the control group over the surveillance period. Analyses were performed using SAS and STATA.

Role of the Funding Source

GSK and CISM both received financial support to undertake the work described in this manuscript from the PATH Malaria Vaccine Initiative (MVI) that was involved in all aspects of the study design and interpretation (as per authorship guidelines). MVI funded this work through a grant from the Bill & Melinda Gates Foundation. Core funding for CISM is provided by the Spanish Agency for International Cooperation (AECI). Results

Results

FIG. **1** shows the trial profile. 326 out of 446 pregnant women fulfilled the eligibility criteria and delivered 329 newborns. Of those, 251 infants were screened and 214 (85%) of the screened children were enrolled in the trial and received the first dose of DTPw/Hib vaccine. 93 (86%) of the children in the RTS,S/AS02D and 92 (85%) in the Engerix-B™ group entered ADI follow-up. During the follow-up, consent was withdrawn from seven children in the RTS,S/AS02D group and eight children in the Engerix-B™ group. Three children in each group received the wrong vaccine at Dose 2 of RTS, S/AS02D or Engerix-B™. They were not included in the ATP analysis. Baseline characteristics for both groups are presented in Table 1.

FIG. **2***c* shows the proportion of all administered doses where a solicited symptom was recorded. The figure presents four groups, as the data for DTPw/Hib and OPV are segregated by randomisation assignment. The proportion of children with pain was high and similar in all groups. The relative proportion of symptoms was similar after each injection of RTS,S/AS02D and comparable in magnitude (data not shown). No grade 3 solicited symptom was documented in the RTS,S/AS02D group, and the incidence was very low in the other groups (data not shown). In the ITT cohort followed until end of follow-up on 6 Mar. 2007, there were 31 SAEs in the RTS,S/AS02D group and 30 SAEs in the Engerix-B™ group. None of them were reported as related to vaccination. There were four deaths during this same follow-up period; all of them after the ADI period had finished at study month 6 (2 in RTSS/AS02D group and 2 in the Engerix-B™ group). All of the deaths occurred at home. Verbal autopsies suggest that one death in the RTS,S/AS02D group was due to septic shock, and the remaining three due to gastroenteritis and severe dehydration. The values and proportion of abnormal haematology and biochemical values after Dose 1 and after Dose 3 were similar in both groups and do not raise any safety signal (data not shown).

For the 214 subjects enrolled in the trial (107 RTS,S/AS02D and 107 Engerix-B™) data for the EPI antigens responses are available for 151 subjects post dose 3 (76 in the RTS,S/AS02D and 75 in the control group). There are no differences in seroprotection/seropositivity and titers between the subjects in the two groups (Aide et al, manuscript in preparation). All but three children reached seroprotective levels against all EPI antigens. These three children will be re immunised with the antigens to which they have failed to respond.

Anti-CS and anti-HBsAg antibody levels measured against CS and HBsAg are shown in Table 2. At screening 24/76 (32%) and 26/77 (34%) of the infants in the RTS,S/AS02D and Engerix-B™ group, respectively, had low titres of detectable anti-CS antibodies. One month after dose 3, 99% (70/71) of infants that received RTS,S/AS02D had detectable anti-CS antibodies, while the corresponding figure among infants that received Engerix-B™ is 4% (3/68). Three and a half months after dose 3 (study month 6), the proportion of anti-CS positives in the RTS,S/AS02D remained high (98%) but the GMT had decreased. The anti-CS GMT in the Engerix-B™ group remained low, even though the prevalence of detectable antibodies increased to 20% (12/61). Response to hepatitis B was good in both groups (Table 2).

FIG. **4** shows the proportion of children with at least one episode of malaria infection during the ADI follow-up starting 14 days after dose 3 of RTS,S/AS02D or Engerix-B™ up to study month 6. A total of 68 new infection were documented during this follow-up period, 22 in the RTS,S/AS02D group and 46 in the Engerix-B™ group. The crude vaccine efficacy (VE) estimate was 62.2% (95% CI 37.1%; 77.3%, p=0.0002) over the 3 month follow-up period. Adjusted by distance to the health centre and community of residence, the VE was 65.9% (95% CI 42.6%; 79.8%, p <0.0001) (Table 3). The point prevalence of infection at study month 6 was similar between the two groups (5% in the RTS,S/AS02D group vs 8% in the control group, p=0.536), nor were there differences between mean parasite densities (2082 parasites per microliter (SD 5604) in the RTS,S/AS02D group vs 2579 (SD 6088) in the control group, p=0.85).

Exploratory endpoints contained in the RAP included efficacy estimates for clinical malaria using different cohorts and case definitions. Efficacy based on an ITT cohort followed from month 0 to month 6, using the primary case definition of malaria (first or only episode of fever with more than 500 parasites per microliter) detected through both ADI and PCD, was 35.5% (95% CI-7.5%; 61.3%, p=0.093). Further efficacy estimates for clinical malaria in an ATP cohort starting 14 days after dose 3 of RTS,S/AS02D or Engerix-B™ up to study visit at 6 months, the same follow-up period used in the primary VE estimate for infection, are shown in table 3. Table 4 gives details of further follow up for 12 months post dose 3, i.e. from 3-14 months.

The relation of anti-CS antibody titres to the risk of malaria was examined in a number of ways. Firstly, we compared anti-CS antibody titres after dose 3 of RTS,S/AS02D or Engerix-B™ in the group of infants where no malaria infection was documented during the follow-up versus those that had at least one episode. On average anti-CS antibody titres were higher in the former group (208 vs. 132, p=0.026). Secondly, the hazard ratio was 71% lower among infants in the higher tertile of the distribution of antibodies than among infants in the lower tertile (95% CI 8.4%; 90.7%, p=0.035). Finally, we examined risk of malaria infection in relation to the increase in antibody titres. Doubling of antibody titres was associated with a reduction in the risk of a new infection of 6.4% (95% CI 10.8; 1.8) p=0.007. A ten times increase in anti-CS titers is associated with a 19.8% reduction in the risk of new infections (95% CI 31.6; 5.9).

Based on an analysis of the results vaccine efficacy for new infections was 65% over a 3 month follow-up period after completion of immunizations. This efficacy estimate is higher than the 45% reduction reported in a previous trial (026 trial) among older children aged 1 to 4 years followed in a comparable ADI system in the same study area (Alonso P L, Sacarlal J, Aponte J J, Leach A, Macete E, Milman J, et al. Efficacy of the RTS,S/AS02A vaccine against *Plasmodium falciparum* infection and disease in young African children: randomised controlled trial. Lancet 2004 Oct. 16-22; 364 (9443):1411-20). The follow-up periods in this trial and the 026 trial were not identical, slightly shorter in the infants than in the older children. Secondly, confidence intervals of the two estimates overlap.

TABLE 1

Baseline characteristics	Engerix-B ™ (n = 92)	RTS, S/AS02D (n = 93)
Age at first dose [weeks]	8.3 (1.0)	8.3 (1.4)
Gender		
Female	53 (58%)	43 (46%)
Male	39 (42%)	50 (54%)
Area		
Ilha Josina	64 (70%)	63 (68%)
Taninga	28 (30%)	30 (32%)
Distance [Kilometers]		
0-5	79 (86%)	77 (83%)
5-10	9 (10%)	9 (10%)
10-17	4 (4%)	7 (7%)

Data are mean (SD) or number of children (%)

TABLE 2

Geometric mean titres (GMT) for Anti-CS and Anti-HBsAg

	Engerix-B ™		RTS, S/AS02D	
Geometric Mean Titre/Timing	n	value (95% CI)	n	value (95% CI)
Anti-circumsporozoite				
Baseline	77	0.4 (0.3; 0.4)	76	0.4 (0.3; 0.5)
30 days after third dose of RTS, S/AS02D or Engerix-B ™	68	0.3 (0.2; 0.3)	71	199.9 (150.9; 264.7)
106 days after third dose of RTS, S/AS02D or Engerix-B ™	61	0.4 (0.3; 0.5)	53	58.8 (41.8; 82.8)
Anti-HBsAg				
Baseline	70	16.6 (11; 25)	72	14 (9.6; 20.5)
30 days after third dose of RTS, S/AS02D or Engerix-B ™	64	392.4 (297; 518.5)	68	10081.6 (7394.9; 13744.4)

TABLE 3

Vaccine efficacy from 14 days after third dose of Engerix-B ™ or RTS, S/AS02D until visit at month 6

	Engerix-B ™			RTS, S/AS02D			Vaccine efficacy		
Outcome	Events	PYAR	Rate	Events	PYAR	Rate		(95% CI)	p
Malaria infection									
First or only episode of parasitaemia >0	46	17.2	2.7	22	21.8	1.0	65.9%	(42.6%; 79.8%)	<0.001
Clinical malaria									
First or only episode of fever and parasitaemia >500 per microliter	22	19.6	1.1	9	22.6	0.4	65.8%	(25.3%; 84.4%)	0.007
First or only episode of fever or history of fever and parasitaemia >0	35	18.2	1.9	17	22.4	0.8	63.1%	(33.6%; 79.6%)	<0.001

PYAR = Person-years at risk. Vaccine efficacy estimates adjusted by distance from health facility and community.

TABLE 4

038 trial Efficacy ATP [over a year post dose 3 i.e. 3 to 14 months]

	RTS, S/AS02D				Engerix-B				Point estimate of VE adjusted
	Subjects (N)	No. of events	PYAR	Rate	Subjects (N)	No. of events	PYAR	Rate	for covariates (%)
Disease 1 [a]	93	36	61.83	0.582	92	45	51.27	0.878	33.122
Disease 2 [b]	93	45	57.26	0.786	92	57	41.34	1.379	41.978
Disease 1 [c]	93	58	72.69	0.80	92	74	68.83	1.08	26.2

	Point estimate of VE adjusted for covariates			Point estimate of VE unadjusted for covariates			
	95% CI		P value	(%)	95% CI		P value
Disease 1 [a]	−4.056	57.017	0.074	34.313	−1.838	57.631	0.060
Disease 2 [b]	13.754	60.965	0.007	41.893	14.044	60.719	0.007
Disease 1 [c]	−16.6	53.3	0.1918	27.2	−14.0	53.6	0.1645

PYAR: Episodes/Person Years at Risk; VE: Vaccine Efficacy (1-HR); CI: Confidence Interval; p value from Cox PH model; Poisson regression for multiple episodes

[a] first or only episodes; the presence of *P. falciparum* asexual parasitemia >500 per μL and the presence of fever ≥37.5° C.

[b] first or only episodes; any level of *P. falciparum* asexual parasitemia and the presence of fever ≥37.5° C. or a history of fever within 24 hours

[c] multiple episodes; the presence of *P. falciparum* asexual parasitemia >500 per μL and the presence of fever ≥37.5° C.

Adjusted estimates for area and distance from health center

ADI and PCD included

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The invention claimed is:

1. A method for inducing an immune response against *Plasmodium falciparum* in an infant comprising

a) selecting an infant from about 1 week old to about 50 weeks old and is at risk of malaria disease or infection; and

b) administering an immunogenic composition comprising

i) a fusion protein comprising a polypeptide comprising a *Plasmodium falciparum* circumsporozoite protein (CS) antigen or an immunogenic fragment thereof comprising substantially all the C-terminal portion of the CS protein and four or more tandem repeats of the CS protein immunodominant region; and

hepatitis B surface antigen (HBsAg), and

ii) a pharmaceutically acceptable adjuvant comprising 3-O-deacylated monophosphoryl lipid A (3D-MPL), and QS21,

wherein the CS antigen is expressed at the pre-erythrocytic stage of malarial infection.

2. A method for treating, preventing or reducing the incidence of clinical malaria in an infant comprising

a) selecting an infant from about 1 week old to about 50 weeks old and is at risk of malaria disease or infection; and

b) administering an immunogenic composition comprising

i) a fusion protein comprising a polypeptide comprising a *Plasmodium falciparum* circumsporozoite protein (CS) antigen or an immunogenic fragment thereof comprising substantially all the C-terminal portion of the CS protein and four or more tandem repeats of the CS protein immunodominant region; and

hepatitis B surface antigen (HBsAg), and

ii) a pharmaceutically acceptable adjuvant comprising 3-O-deacylated monophosphoryl lipid A (3D-MPL), and QS21,

wherein the CS antigen is expressed at the pre-erythrocytic stage of malarial infection.

3. The method of claim **2**, wherein the risk of developing clinical symptoms of malaria is reduced.

4. The method of claim **3**, wherein the malaria is non-severe malaria.

5. The method of claim **2**, wherein the immunogenic composition further comprises one or more additional antigens expressed on the sporozoite or pre-erthrocytic stage of malarial infection or an immunogenic fragment thereof.

6. The method of claim **2**, wherein the additional antigen is selected from the group consisting of liver stage antigen-1 (LSA-1), liver stage antigen-3 (LSA-3), apical merozoite antigen-1 (AMA-1), merozoite surface protein-1 (MSP-1), thrombospondin related anonymous protein (TRAP) and Exp-1.

7. The method of claim **2**, wherein the CS antigen comprises substantially all the C-terminal portion of the CS protein of *Plasmodium falciparum*, four or more tandem repeats of the CS protein immunodominant region, and the surface antigen from hepatitis B (HBsAg).

8. The method of claim **2**, wherein the fusion protein is RTS.

9. The method of claim **8**, wherein the immunogenic composition comprises RTS and S to form a composition of mixed particles RTS,S.

10. The method of claim **9**, wherein the amount of RTS,S is 25 μg per dose.

11. The method of claim **2**, wherein the adjuvant further comprises an oil in water emulsion.

12. The method of claim **2**, wherein the adjuvant further comprises liposomes.

13. The method of claim **2**, wherein the immunogenic composition is formulated as a pediatric dose.

14. The method of claim **13**, wherein the pediatric dose is administered once, twice or three times.

15. The method of claim **14**, wherein the pediatric dose is administered at two, three, four or five week intervals.

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